Rec'd PCT/PTO 20 JUN 2005
IN03/316

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GOVERNMENT OF INDIA
MINISTRY OF COMMERCE & INDUSTRY,
PATENT OFFICE, DELHI BRANCH,
W - 5, WEST PATEL NAGAR,
NEW DELHI - 110 008.

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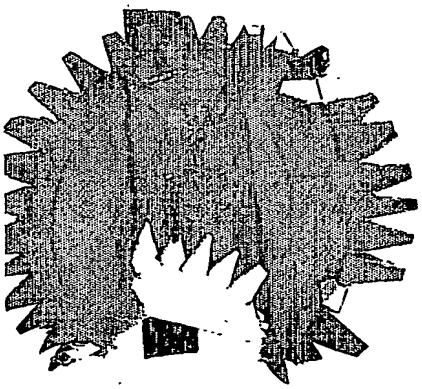
I, the undersigned, being an officer duly authorized in accordance with the provision of the Patent Act, 1970 hereby certify that annexed hereto is the true copy of the ***Application*** *and* ***Provisional Specification*** *filed in connection with Application for Patent No.* ***1283/Del/02*** *dated* ***19th December 2002.***



Witness my hand this 29th day of September 2003.

(S.K. PANGASA)
Assistant Controller of Patents & Designs

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1283-02

FORM-1

(To be filed in Triplicate)

THE PATENTS ACT, 1970

19 DEC 2002

(39 of 1970)

APPLICATION FOR GRANT OF A PATENT

[See Sections 5(2) 7, 54 and 135)

1. We 1) DEPARTMENT OF BIOTECHNOLOGY, of Block 2, 7th Floor, CGO Complex, Lodi Road, New Delhi-110 003;
2) DECCAN COLLEGE OF MEDICAL SCIENCES AND ALLIED HOSPITALS, of Kanchanbagh, Hyderabad-500 058.

2. hereby declare-

(a) that ~~I am/~~We are in possessin of an Invention titled
A PROCESS OF CHARACTERIZATION OF HEPATOCYTES FROM GOAT

Title

(b) that the Provisional /~~Complete~~ Specification relating to this invention filed with this application.

PUBLICATI

(c) that there is no lawful ground of objection to the grant of a patent to me / us.

3. Further declare that the inventor(s) for the said invention is / are :

Surname first and then name of inventor/s

1) VENKATESHAN VIJAYLAKSHMI, (2) BEGUM NASEEM,
3) KHAN ALEEM AHMED, (4) CAPOOR ADARSH KUMAR
5) HABEEB MOHAMMED AEJAZ, (6) HABIBULLAH CHITTOOR MOHAMMED,
of Centre For Liver Research and Diagonostics
Deccan College of Medical Sciences and Allied Hospitals
Kanchanbagh, Hyderabad-500 058, all are Indian nationals.

4. I/We, claim the priority from the application(s) filed in convention countries, particulars of which are as follows :

NA

5. I/We state that the said invention is an improvement in or modification of the invention the particulars of which are as follows and of which I/We are the application/patentee:

NA

6. I / We state that the application is divided out of my/our application, the particulars of which are given below and pray that this application be deemed to have been filed on.....NA..... under section 16 of the act.

7. That I am / We are the assignee of the true and first inventors.

8. That my / our address for service in India is as follows :
L S DAVAR & CO., of 5/1, 1st Floor, Kalkaji Extension, New Delhi-110 019 and
Monalisa, Flats IB & IC, 17, Camac Street,
Kolkata-700 017.
Phones : 247-3996, 247-5918, 280-5536
Fax No. : 91-33-247-5886, 240-6292
91-11-646-4443

9. **Following declaration was given by the inventor(s) or applicant(s) in the convention country :**

I / We the true and first inventors for this invention or the applicant(s) in the convention country declare that the applicant(s) herein is / are my / our assignee or legal representative.

Signature of the true and first Inventor/s or Applicant In the convention country with date, name to be given below Signature

1. VENKATESHAN VIJAYLAKSHMI
2. BEGUM NASEEM
3. KHAN ALEEM AHMED
4. CAPOOR ADARSH KUMAR
5. HABEEB MOHAMMED AEJAZ
6. HABIBULLAH CHITTOOR MOHAMMED

10. That to the best of my / our knowledge, information and belief the fact and matters stated herein are correct and that there is no lawful ground of objection to the grant of patent to me / us on this application.

11. Following are the attachment with application :

(a) Provisional/~~Complete~~ specification (3 copies).
(b) Drawings (Sheets) 3 copies. NA
(c) Priority document/s NA
(d) Statement and undertaking on Form 3 in dupl.
(e) Form 5. NA
(f) Power of Authority. To Follow
(g)
(h)
(i) Fee Rs. ~~1,500/-~~/Rs. 5,000/- in cheque / bank draft. bearing No.....date..... on.....Bank.

To be Signed by applicant or authorised patent agent

I/We request that a patent may be granted to me/us for the said invention.

Dated this.....16th.....day of.....December.....2002

Signature (/. BANERJEE)

OF L. S. DAVAR & Co.
APPLICANTS' AGENT

To
The Controller of Patents
The Patent Office
at New Delhi

1283-02

FORM-2 19DEC 2002

THE PATENTS ACT, 1970

(39 of 1970)

PROVISIONAL/~~COMPLETE~~

SPECIFICATION

SECTION 10

ORIGINAL

TITLE

A PROCESS OF CHARACTERIZATION OF HEPATOCYTES FROM GOAT

APPLICANT

1) DEPARTMENT OF BIOTECHNOLOGY, of Block 2, 7th Floor CGO Complex, Lodi Road, New Delhi-110 003;
2) DECCAN COLLEGE OF MEDICAL SCIENCES AND ALLIED HOSPITALS, of Kanchanbagh, Hyderabad-500 058

The following specification particularly describes the nature of the invention and the manner in which it is to be performed

FIELD OF THE INVENTION

This invention relates to a process of characterization of hepatocytes from goat.

BACKGROUND OF THE INVENTION

FHF is a clinical syndrome associated with a very high mortality rate, for which no satisfactory therapy is currently available, other than liver transplantation. FHF is a true medical emergency, which requires immediate therapeutic intervention. The treatment of FHF has been difficult due to the complex nature of physiological functions performed by the liver. Liver transplantation is the only approach to improve survival in these patients. However, liver transplantation is a major surgical procedure, is very expensive, and requires prolonged immunosuppression. It can be performed only at select advanced centers by highly skilled surgeons. Procurement of donor organs is also a major problem due to the shortage of human donors.

Hepatocyte transplantation appears to be an alternative to liver transplantation. Studies have been carried out to assess the efficacy of hepatocyte transplantation in acute liver failure in animal models such as rats, mice, and pigs, and have shown encourgaging results (Sommer et al. 1979, Kawai et al. 1987, Gagandeep et al. 2000, Arkadopoulos et al. 1998, Allen and Soriano 2001, Malhi and Gupta 2001). Investigators have also demonstrated improvement in survival in human patients with FHF with allogeneic hepatocyte transplantation (Habibullah et al. 1994, Bilir et al. 2000). The data emerging from these experimental studies justifies the use

of hepatocyte transplantation as a substitute to orthotopic liver transplantation.

The different sources of hepatocytes are human cadaver, human fetus and higher animals. Severe shortage of human cadaver livers, limits their use for therapeutic purpose. Human fetal hepatocytes from aborted or medically terminated pregnancies can be used, as fetal hepatocytes are less immunogenic, exhibit differentiated functions including albumin synthesis, bile formation, and urea cycle activity, and may provide metabolic support in FHF (Baver et al. 1991, Rehman et al. 1993, Habibullah 1992, 1997). Habibullah et al. (1994) have reported that, intraperitoneal transplantation of human fetal hepatocytes resulted in the recovery of 3 out of 7 patients with acute liver failure. However the use of human fetal cells for transplantation is limited, as they are required in large numbers for a single transplantation.

The shortage of human donor organs has focused interest in the use of xenogeneic organs/cells for transplantation. Clinical interest in xenotransplantation (XT) and scientific research in this field have increased enormously (Reemtsma 1991). XT is considered promising because if it is successful, it can combat the shortage of human donor organs. The lives of many patients needing organ transplants may possibly be saved.

Pig xenografts have been considered as an alternative source of organs for transplantation (Niekrasz et al. 1992, Sach 1994). However, the use of pig organs/cells for human therapy has certain limitations. i) The presence of alpha-gal epitope (Gal alpha 1-3Gal beta 1-4GlcNAc-R) abundantly on pig cells, and its interaction with the human natural anti-Gal antibody, is considered to be the major obstacle in the way of pig to human

xenotransplantation (Galili 1993). Incubation of pig cells expressing α-gal epitopes with human serum was found to induce complement mediated lysis of the cells as a result of the binding of anti Gal IgM molecules to α-gal epitope, followed by activation of complement (Good et al. 1992, Oriol et al. 1993, Sandrin et al. 1993). Studies in monkeys have further indicated that, invivo binding of anti-Gal to α-gal epitopes on endothelial cells of pig xenografts results in complement mediated lysis of these cells, with ensuing collapse of the vascular bed and hyperacute rejection of the xenograft (Collins et al. 1994). ii) The risk of transmission of porcine endogenous retrovirus (PERV) to the recipients is also a major concern (Allan 1996, Patience et al. 1997, Speck et al. 2001).

OBJECTS OF THE INVENTION

An object of this invention is to isolate the hepatocytes from goat.

Another object of this invention is to characterize the isolated cells to demonstrate the hepatic functions.

Further object of this invention is that the process is efficient and cost effective.

At the out set of the description which follows, it is to be understood that the ensuing description only illustrates a particular form of this invention. However, such a particular form is only intended as an exemplary embodiment and teaching of the invention and not intended to be taken restrictively.

BRIEF DESCRIPTION OF THE INVENTION

According to this invention there is provided a process for characterization of hepatocytes cells in goat comprising in the steps of isolating the cells from goat.

Treating such isolated cells in a buffer medium and then subjecting it to a set up of characterization.

In accordance with this invention the hepatocytes are isolated and characterized.

Isolation of hepatocytes:

The animals were sacrificed and livers were collected in cold and sterile conditions. They were processed for the isolation of hepatocytes in a laminar flow unit under aseptic conditions. The liver was perfused extensively with Hank's buffer through the portal vein, using a polythene catheter, until it became colourless. Collagenase solution was passé into the liver and incubated at room temperature. The liver was cut into small pieces and kept on constant stirring for 20 minutes. It was then sieved to remove connective tissue. Cell suspension was collected; Hank's medium containing Ca^{2+} and Mg^{2+} salts was added and kept for gravity sedimentation. The supernatant contining RBCs and dead or broken cells was removed. The cell suspension was washed 4-5 times with Hanks medium, till hepatocyte suspension was obtained. The cells were then suspended in a medium . The cell yield and viability were checked and the cells were stored at 4°C.

Staining:

They were decolourised in alcohol. They were kept on blotting paper and then stained. They were given two changes of the slides each followed by two changes of xylol minutes each.

Characterization:

The percentage of viable cells was calculated as the number of cells unstained divided by total number of cells (stained + unstained cells) X 100.

MTT Assay:

MT assay was done by the method of Mossman 1983 with slight modification. Cells were taken and centrifuged. To the cell pellet, MTt reagent was added and incubated. The cell suspension was centrifuged and to the cell pellet. isopropanol was added and kept at room temperature. The purplish blue colour of the supernatant was read at 540nm. The amount of formazan formed was expressed as µM formazan/10^6 cells.

MEMBRANE INTEGRITY:

Hepatocyte suspension was centrifuged and the supernatant was separated. The pellet was solubilized by adding solubilizing solution as described by Mamprin et al. (1995). The LDH activity was determined in the pellet and in the supernatant by using a commercial LDH kit (E. Merck India Ltd.). To reaction solution taken in different curvets sample (supernatant, cell lysate) were added. They were mixed, incubated for 1 minute and absorbance was read at 340nm. The change in absorbance was measured every minute. Percentage of LDH leakage was calculated as the ratio of percentage LDH activity in the supernatant to the total percentage LDH activity in supernatant to the total percentage LDH activity in supernatant and cell pellet.

Na^+K^+ ATPase Activity:

Na^+K^+ ATPase activity was estimated by the method of Sawa and Gilbert (1981). The hepatocytes were homogenized in sucrose using glass-teflon homogeniser and the Na^+K^+ ATPase was estimated in the final reaction mixture of (1) Nacl, KCl, $MgCl_2$, EDTA, Imidazole and oubain at pH 7.4. The assay was carried out with protein. The reaction was started by adding ATP and incubating minutes. The reaction was terminated and the inorganic phosphate (Pi) was determined from the supernatant. Enzyme activity was calculated as the difference in the Pi content between the total ATPase and Mg^{2+} ATPase activity.

Estimation of phosphorous:

Phosphorus was estimated by the method as described by Raghuramulu et al. (1983). To test sample, reagent C was added, mixed and incubated. This was allowed to attain room temperature and the absorbance was read against the blank. A calibration curve of phosphorus was done and the activity of Na^+K^+ ATPase was expressed as µM Pi/hr/mg protein.

Lipid Peroxidation:

Malondialdehyde was estimated by the method of Ohkawa et al. (1979), with slight modifications. Cell suspension ($5x10^6$ cells) was added to Sodium dodecyl sulphate, acetic acid and the pH of the solution was adjusted with NaOH. To this aqueous solution of 2-thiobarbituric acid was added. The volume was made up to 4 ml with distilled water and then heated in boiling water bath for 60 minutes. The tubes were capped with marbles to prevent condensation. After cooling under tap water, 1 ml of distilled water and the mixture of n-butanol and pyridine (15:1) was added and shaken

vigorously. The tubes were then centrifuged and the organic layer was aspirated out. and its absorbance at 532 nm was measured in a colorimeter. The levels of lipid peroxides were expressed as n moles of MDA formed/mg of protein.

Detoxification:

Cells were incubated with ammonium chloride. At the end of 1 hour, supernatant was collected and the amount of urea formed was estimated by a commercial kit (E.Merck India Limited). To 1 ml of reaction solution taken in different tubes (blank, standard, test), distilled water, urea standard, and sample were added respectively. They were mixed, and incubated. Absorbance. (A1) was measured at 340nm. They were again incubated exactly after 60 seconds, and absorbance (A2) was measured separately for each tube at 340nm, and the amount of urea formed was calculated and expressed as mM urea/10^6 cells.

Cytochrome P450 activity:

Cytochrome P450 activity was measured using diazepam as substrate by HPLC method.

The HPLC system consisted of Waters LC Module-1 (Water's Milford MA, USA) and the detector used as photodiode array detector (PDA). Each incubation mixture in a total volume of 0.25ml contained 0.125ml potassium phosphate buffer (pH 7.4), 0.1ml ($4x10^6$) hepatocytes, Diazepam and NADPH. Reaction was terminated with methanol. Incubation mixture without Diazepam served as blank. For the purpose of estimation of Diazepam in samples, a similar incubation mixture spiked with Diazepam was terminated immediately which served as standard. After terminating the

reaction and centrifuging. Organic phase (1ml) was separated and evaporated to dryness under nitrogen. The residue was reconstituted with mobile phase and was injected into HPLC. The mobile phase was pumped through the stationary phase at a flow rate of 1ml/min. The eluent was monitored for metabolites (Oxazepam and desmethyl diazepam) and drug (diazepam) using a PDA detector operating at 247 nm. Under these conditions, the retention times for metabolites (Oxazepam and desmethyl diazepam) and drug. The activity of the enzyme was expressed as the percentage of diazepam disappeared.

Glutathione-S-transferase (GST) activity:

GST activity was measured by the method of Habig et al. (1974). To 1.0 ml of phosphate buffer, 0.1 ml CDNB and 0.1 ml of cytosolic fraction were added. The volume was adjusted with distilled water. The reaction mixture was pre-incubated. The reaction was started by the addition of 0.1 ml of glutathione solution and the absorbance was followed at 340nm (Beckman DU 640 B sprectrophotometer). The reaction mixture without cytosolic fraction was used as a blank. Activity of the enzyme was expressed as µmol CDNB-GSH conjugate/mg protein.

Estimation of Protein:

Protein was estimated by the method of Lowry et al. (1951). To 100 µl of test sample, distilled water was added. 5ml of solution (alkaline copper sulphate reagent) was added, mixed and kept at room temperature. To this diluted Folin reagent was added, mixed well and incubated at room temperature for colour development. The absorbance was read at 680 nm. BSA calibration curve was done using 10, 20, 30, 40, 60, 80, 100 µg of

protein. The final concentration of protein was expressed as µg/ml of cell lysate.

Dated this 16 th day of DECEMBER 2002.

of L S DAVAR & CO.,
Applicants' Agent

PCT Application
IN0300316

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